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An efficient preparation of chondroitin sulfate and collagen peptides from shark cartilage

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Abstract

Chondroitin sulfate and collagen peptides were extracted with highest yield from shark cartilage under the following conditions: milling for 6 min; proteolysis with neutrase at 45°C for 5 h; enzyme to substrate ratio of 1800 U/g; solvent to material ratio of 10 mL/g. They were then separated on a column of DEAE-Sepharose Fast-flow. Most chondroitin sulfate (88.4% of total) and collagen peptides (78.3% of total) were recovered. The peptides were characterized by predominance of glycine, proline and hydroxyproline which made up around half of the total amino acid content, and their molecular weights were more than 1000 Da, but less than 4186.6 Da. The method has several advantages for higher quality of chondroitin sulfate, low environmental impact and recovery of collagen peptides.

Introduction

Cartilage is rich in glycosaminoglycans (10-40% by dry weight) and type II collagen (25-55% by dry weight), both of which can be converted to highly profitable products (Garnjanagoonchorn *et al.*, 2007). Chondroitin sulfate (CS) as a major glycosaminoglycan is composed of N-acetyl-D-galactosamine and D-glucuronic acid sulfated at positions 4 or 6 (Jackson *et al.*, 1991). It has been reported to have a wide range of applications in the pharmaceutical, cosmetic, and food industries for its anti-degenerative arthritis, anti-inflammation, antiatherogenic, antitumor and hypolipidemic capacities (Albertini *et al.*, 1999; Campo *et al*., 2004). Collagen hydrolysate is another active component prepared by hydrolysis of denatured collagen. It has been extensively used in such consumables as cosmetics, nutraceuticals and dietary supplements (Langmaier *et al.*, 2002; Morimura *et al*., 2002).

Presently, various techniques have been developed to extract (Park *et al*., 2001; Jo *et al.*, 2005; Cheng *et al*., 2011) and analyze CS (Volpi *et al*., 2008; Gargiulo *et al*., 2009), but not much attention has been paid to the economical production of CS and collagen hydrolysate. Usually, in order to purify CS, large amounts of collagen peptides were denatured by chemicals (e.g. trichloroacetic acid) before being discarded. This resulted in an underutilization of cartilage and generation of waste (Garnjanagoonchorn *et al.*, 2007; Im *et al*., 2009). Thus, the utilization of cartilage resources, especially the scarce shark cartilage, with more intelligence and foresight is needed.

In this study, various factors affecting enzymatic digestion were investigated. The enzymatic extract was further chromatographed on a column of DEAE-Sepharose Fast-flow to separate CS and collagen peptides. Furthermore, the characteristics and results of this method were compared with conventional method.

Materials and Methods

Material and reagents

Dried cartilage of blue shark (Prionace glauca) was provided by Jiaxing Hengjie Biophamaceutical Co., Ltd. (Zhejiang, China) and cut into pieces of approximately 2 × 4 cm. Standard CS from shark cartilage (assay ≥ 90%, USP standards) was purchased from Sigma-Aldrich (U.S.A.); molecular weight (MW) standard exenatide acetate (4186.6 Da) was obtained from GL Biochem Co., Ltd. (Shanghai, China). Neutrase (2.0×10^5 U/g) was obtained from Imperial Jade Bio-technology Co., Ltd. (Ningxia, China); alcalase (2.0×10^5 U/g), papain (6.5×10^5 U/g), bromelain (5.0×10^5 U/g) and pancreatic enzyme (4.0×10^3 U/g) were provided by Guangxi Nanning Pangbo Biological engineering Co., Ltd. (Guangxi, China); acid protease (5.0×10^4 U/g) was obtained from Jienuo Enzyme Co., Ltd (Shandong, China). All other reagents were analytical grade.

Extraction and purification of CS and collagen peptides

Before extraction, the shark cartilage was ground

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with a vibration mill (model XDW-6J; Dawei Machinery Co., Ltd., Jinan, China) equipped with a water-cooled drum and steel rods as grinding bodies (18 mm diameter, 174 mm length, the mass ratio of total grinding bodies to load = 100:1) for maximum 6 min to obtain a fine powder. Further increase of grinding time was not suitable as it would result in agglomeration of particles in water and even shear degradation of protein. The cartilage powder (10.0 g) was then digested under the following conditions: different enzymes such as neutrase, alcalase, papain, bromelain and acid protease and their optimum pH values and temperatures; 4 h; enzyme to substrate ratio of 2000 U/g; solvent to material ratio of 10 mL/g. After proteolysis, enzymatic reaction was terminated by heating at 70°C for 15 min. The digest was centrifuged (6000 rpm) at room temperature for 30 min and the supernatant was the enzymatic extract. The content (mg/mL) of CS and hydroxyproline in extracts was determined by cetylpyridinium chloride (CPC) and Woessner methods, respectively. Then the effects of enzyme to substrate ratio, solvent to material ratio, temperature and extraction time on the extraction rates of CS and collagen peptides were determined successively.

The enzymatic extract was adjusted to pH 4.0 with 6 M HCl and loaded onto a column containing 50 mL of DEAE-Sepharose Fast-flow resin (Zhejiang Zhengguang Industrial Co., Ltd.). The flow through was collected. The column was initially washed with 50 mL of water at a flow rate of 2 mL/min, and the wash-unbound fraction was carefully collected and combined with the flow through. It was then dialyzed (MWCO 1000 Da) against water for 2 days and freeze-dried to obtain collagen peptides. Hydroxyproline content in the solutions before and after resin treatment were determined to estimate the percent recovery for collagen peptides. CS, retained by the resin, was eluted with 265 mL of 1.0 M NaCl solution at a flow rate of 1 mL/min. The eluate was then precipitated with three volumes of 95% (v/v) ethanol, and dried in a stove at 105°C for 30 min. The percent recovery of CS was determined by comparing its content in the eluate with that in the extract.

Conventional method of producing CS

The unground shark cartilage (10.0 g) was extracted with 100 mL of boiling water for 6 h, and then digested by pancreatic enzyme at pH 8.5 and 53°C for 6 h. Enzymatic reaction was terminated by acidification to pH 6 and heating at 70°C for 15 min. After centrifugation (4000 rpm/10 min), the supernatant was precipitated with two volumes of 95% (v/v) ethanol. The sediment was dissolved in hydrogen peroxide: water (1:100 v/v) and hydrolyzed at pH 10 and 60°C for 4 h. After centrifugation (4000 rpm/10 min), the supernatant was precipitated with three volumes of 95% (v/v) ethanol and dried in a stove at 105°C for 30 min.

Analytical methods

CS content in raw material was determined by the carbazole assay (Bitter and Muir, 1962). CS content in the enzymatic extract and collected fractions were analyzed using the CPC method (Liang *et al*., 2002) with a titrator (DL50, Mettler-Toledo Instruments Inc., Switzerland). CS in the finished product was analyzed by a Waters HPLC system equipped with an Atlantis®dC18 column (4.6 × 150 mm, 5 μm, Waters, USA) at 25°C eluted by mobile phase CH_3CN and 1% phosphate buffer (10: 90, v/v) at a flow rate of 0.6 mL/min. The detection wavelength was set at 195 nm. Retention time for CS was approximately 2.99 min. Quantification of protein in the CS product was performed by the Lowry method (Lowry *et al.*, 1951).

The content of hydroxyproline (reflecting that of collagen peptides) was determined according to the method of Woessner (Woessner, 1961) after hydrolysis in 6 M HCl at 110°C for 24 h. Amino acid was analyzed as previously described (Cui *et al.*, 2007). The collagen peptides and raw material were hydrolyzed with 6 M HCl at 110°C for 24 h, and then analyzed by a Hitachi amino acid analyzer L-8900 (Hitachi, Tokyo, Japan). The total nitrogen content of collagen peptides was determined by Kjeldahl analysis. To characterize the average MW and homogeneity of collagen peptides, size exclusion chromatography was performed on a TSK-GEL G2000SWXL column (7.8 × 300 mm, 5 μm, TSK, Japan). Acetonitrile/0.1% trifluoroacetic acid in water (45: 55, v/v) was applied at a flow rate of 0.8 mL/min. The UV absorption was measured at 214 nm and the system was operated at 25°C. All experiments were replicated three times. The data were subjected to one-way analysis of variance using SPSS statistical software, release 17.0 (SPSS Inc., Chicago, IL). Duncan's new multiple range test was used to determine significant differences at the level of $P < 0.05$.

Results and Discussion

Extraction of CS and collagen peptides

The first experiment was carried out to determine the effects of enzyme, enzyme to substrate ratio, solvent to material ratio, temperature and extraction time on the extractability of CS and collagen peptides.

Table 1. Characteristic features of investigated methods

	Pancreatic enzyme-H_2O_2 hydrolysis	Neutrase-anion-exchange chromatography
Pretreatment before proteolysis (h)	6	0.1
% of total CS recovered	86.9 ± 1.5	88.4 ± 0.9
% of total collagen peptides recovered	/	78.3 ± 1.3
CS content (%)	79.5 ± 0.6	98.9 ± 0.4
Protein content in CS (%)	4.8 ± 0.1	1.5 ± 0.1
Total nitrogen content in collagen peptides (%)	/	15.2 ± 0.4

Table 2. Amino acid composition of shark cartilage and collagen peptides

Amino acid	Number of residues/1000	
	Shark cartilage	Collagen peptides
Aspartic acid	55	56
Threonine	29	29
Serine	40	34
Glutamic acid	95	96
Glycine	272	307
Alanine	85	90
Cystine	3	0
Valine	29	26
Methionine	17	11
Isoleucine	47	18
Leucine	45	39
Tyrosine	9	3
Phenylalanine	18	16
Lysine	23	19
Histidine	8	7
Arginine	51	53
Proline	103	116
Hydroxyproline	70	80
Total	1000	1000

The plant-derived and microbial enzymes such as neutrase, alcalase, papain, bromelain and acid protease were studied at their optimal activation pH values and temperatures considering the frequent occurrences of animal epidemics. Figure 1A showed that enzymatic digestion of cartilage with neutrase achieved the highest extraction rates of CS and collagen peptides (94.1 ± 1.6% and 84.5 ± 1.6%, respectively), while bromelain resulted in the lowest extraction rates (80.3 ± 0.5% and 28.5 ± 0.7%, respectively). Figure 1B showed that the extraction rates increased with the concentration of neutrase (up to 1800 U/g) and reached a plateau thereafter. The extraction rates were highest at 10 mL/g (Figure 1C) and 45°C (Figure 1D) for 5 h (Figure 1E) and approximately 90% of total CS and 80% of total collagen peptides were extracted. The enzymatic extract was then chromatographed on DEAE-Sepharose Fast-flow to separate collagen peptides and CS that could be adsorbed to cationic groups of the stationary phase. Water was first used to yield collagen peptides, which usually fail to bind to the resin in the pH range of 4-6 (Whitelock and Iozzo, 2002). Hydroxyproline was detected primarily in the flow-through and wash-unbound fractions, 96.1 ± 1.3% of the loaded amount. CS could not be detected. 1.0 M NaCl solution was then used to yield CS and 93.5 ± 2.5% of the loaded was recovered in the eluate.

Compared to the conventional method, the method described above was attractive (Table 1). It reduced the consumption of ethanol and generation of waste and collagen peptides could be extracted as a useful co-product. This is of economic importance

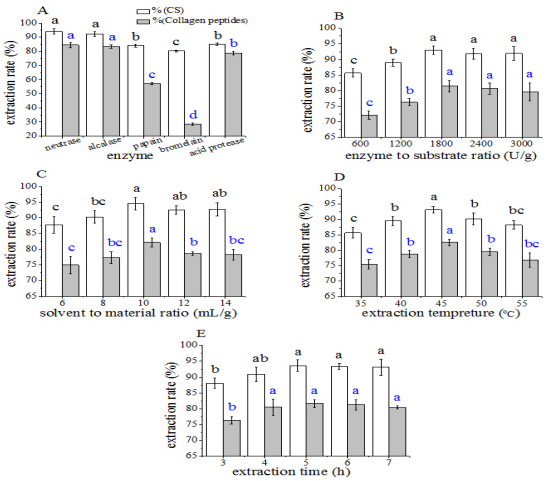

Figure 1. Factors affecting extraction rates of CS and collagen peptides. Extraction rate (%) = mass of CS (collagen peptides) in extracts/mass of CS (collagen peptides) in shark cartilage × 100. Different lower case letters indicate significant differences within treatments. (A) Effect of enzyme (enzyme to substrate ratio of 2000 U/g, solvent to material ratio of 10 mL/g, 4 h, optimum pH and temperature); (B) Effect of enzyme to substrate ratio (solvent to material ratio of 10 mL/g, 45°C, 4 h, neutrase); (C) Effect of solvent to material ratio (enzyme to substrate ratio of 1800 U/g, 45°C, 4 h, neutrase); (D) Effect of temperature (enzyme to substrate ratio of 1800 U/g, solvent to material ratio of 10 mL/g, 4 h, neutrase); (E) Effect of extraction time (enzyme to substrate ratio of 1800 U/g, solvent to material ratio of 10 mL/g, 45°C, neutrase).

in industry and will be helpful in resolving the environmental concerns. For ground cartilage, 6 min of mechanical pretreatment were required before a complete enzymatic digestion, while the time was extended to 6 h at 100°C for unground cartilage. Mechanical pretreatment would induce physical, chemical and physicochemical changes, such as modification of rheological properties, size reduction and a simultaneous increase in the specific surface area (Zhao *et al.*, 2011). These changes, in turn, would eliminate diffusional limitations to a significant extent and accelerate the extraction process. Furthermore, the CS content (Figure 2) related to dried finished product was 1.24 times of that using conventional method and the protein content in CS was apparently lower.

Amino acid composition and average MW of collagen peptides

As stated in Table 2, the collagen peptides were rich in glycine, proline, glutamic acid, alanine and hydroxyproline, which made up around 70% of the total amino acid content. Glycine was the most abundant amino acid and the content was slightly lower than that in shark cartilage collagen (316

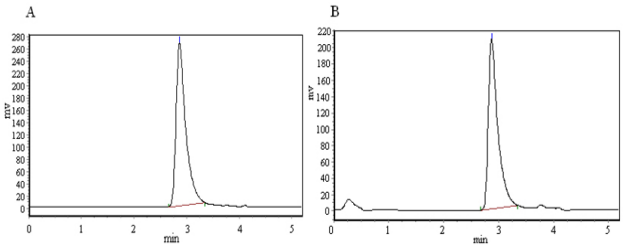

Figure 2. Analytical HPLC chromatograms of CS obtained by (A) neutrase-DEAE-Sepharose chromatography; (B) pancreatic enzyme-H_2O_2 hydrolysis.

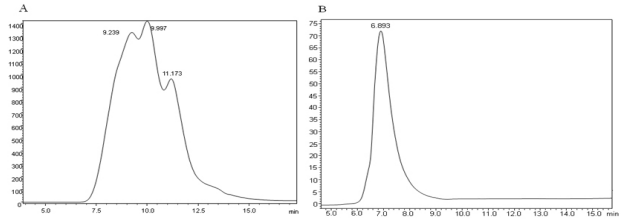

Figure 3. Size-exclusion chromatographic profiles of (A) collagen peptides; (B) exenatide acetate (MW: 4186.6 Da).

residues) (Kittiphattanabawon *et al*., 2010). The content of glycine, proline and hydroxyproline was apparently higher than that in raw material. A loss of isoleucine, serine, methionine, leucine and tyrosine was observed and cystine was absent, mainly due to removal of small peptide fragments and free amino acids during dialysis. As shown in Figure 3, the collagen peptides was eluted later than the MW standard and exhibited three main peaks, suggesting that it was a peptide mixture with the average MWs greater than 1000 Da, but less than 4186.6 Da.

Conclusion

A simple low-cost method was developed to produce CS and collagen peptides from shark cartilage. The optimized parameters for enzymatic extraction were as follows: neutrase; enzyme to substrate ratio of 1800 U/g; solvent to material ratio of 10 mL/g; 45°C and 5 h. 93.5 ± 2.5% of CS was recovered in a 1.0 M NaCl eluate and 96.1 ± 1.3% of collagen peptides in the unbound fractions of DEAE-Sepharose Fast-flow. The main advantages of the proposed method were: (1) recovery of collagen peptides; (2) lessening the environmental impact; (3) high efficiency and (4) higher quality of CS. Further studies are needed to explore methods to manufacture more value-added co-products.

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